The Role of Pyruvate in Neuronal Calcium Homeostasis

EFFECTS ON INTRACELLULAR CALCIUM POOLS*

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It has long been known that pyruvate is essential for survival of prenatal neurons in culture. To understand the role of exogenous pyruvate in neuronal calcium homeostasis, we have investigated the effects of pyruvate (plus malate) addition to dissociated adult rat hippocampal and cerebral cortex cells and cultured CNS neurons having an unrestricted glucose supply. We found that pyruvate (plus malate) increased the respiration rate while ATP levels were unchanged. At the same time, cytosolic free calcium concentrations, $[Ca^{2+}]_i$, decreased while total $^{45}Ca^{2+}$ and $^{40}Ca^{2+}$ accumulation increased. The extra Ca^{2+} accumulated by the cells is attributable to an increase in the size of the intracellular calcium pools. Two such pools were identified on the basis of their sensitivity to specific drugs. The first pool was mobilized by thapsigargin plus tert-butyl hydroquinone and caffeine while the second pool was discharged by the mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (plus oligomycin). The two pools represented about 15–20% and 15–30%, respectively, of the rapidly exchangeable $^{45}Ca^{2+}$ pools in cerebral cortex cells. In cultured hippocampal neurons, the collapse of the mitochondrial membrane potential (as induced by uncouplers (FCCP) or respiratory chain inhibitors (antimycin) caused a large increase in $[Ca^{2+}]_i$ which varied in size and shape among cells and was reduced by external Ca^{2+} chelation. The latter condition also resulted in a partial discharge of FCCP-releasable $^{45}Ca^{2+}$. The effects of FCCP did not result simply from ATP depletion since incubation in glucose-free medium and sequential additions of 2 mM deoxyglucose and 10 μM oligomycin, conditions that led to a dramatic reduction in cellular ATP levels, did not abolish the FCCP-induced $[Ca^{2+}]_i$ rise. Taken together, the results indicate that mitochondria harbor a significant proportion of cellular Ca^{2+}. The sensitivity of the mitochondrial pool size to pyruvate (plus malate) questions previous hypotheses concerning a kinetic limitation for Ca^{2+} accumulation in mitochondria in resting neurons.

The trophic requirements of tricarboxylic acid intermediates in various cell types growing in glucose-containing medium have been known for a number of years (Herzenberg and Roosa, 1960). Lactic acid has been shown to be an autocrine stimulatory molecule essential for the proliferation of immortalized B lymphocytes growing in serum-free conditions in the presence of glucose and glutamine (Pike et al., 1991). Exogenous pyruvate is essential for the survival of prenatal neurons, and it has been suggested that the glial cell population is the most likely candidate for external source of pyruvate and other citric acid cycle intermediates such as α-ketoglutarate, oxalacetate, or amino acids that transaminate to them (Selak et al., 1985; Kaufman and Driscoll, 1992; Facci et al., 1985; Shank and Campbell, 1984). In fact, high affinity transport systems for L-malate and α-ketoglutarate have been reported in synaptosomes (Shank and Campbell, 1984). These metabolites serve to replace molecules that are utilized to replenish the amino acid neurotransmitter pools (Hertz, 1989) but also may be needed by the neurons in order to meet energy requirements which glucose alone cannot satisfy. One of these energy requirements appears to be oxidation in the tricarboxylic acid cycle since the ability to decarboxylate these metabolites by neurons correlates with the ability to survive in their presence (Facci et al., 1986).

It is known that various neuronal trophic factors have long term effects on cell Ca^{2+} homeostasis. Ciliary ganglion cells which suffer developmental death in the embryo will survive in culture medium supplemented with depolarizing concentrations of potassium that promote a sustained increase in intracellular Ca^{2+} (Collins et al., 1991). Sympathetic neurons which die in vitro after acute nerve growth factor withdrawal may also be rescued from death by depolarizing agents that increase $[Ca^{2+}]_i$[1] (Koike and Tanaka, 1991). On the other hand, if $[Ca^{2+}]_i$ levels are too high, for example in response to excitatory amino acids or to the continuous presence of the Ca^{2+} ionophore A23187, neurons will die (Milles and Kater, 1990; Choi, 1987; Mattson et al., 1989), and growth factors can reduce both neuronal death and the rise in $[Ca^{2+}]_i$ caused by glutamate (Matt-

[1] The abbreviations used are: $[Ca^{2+}]_i$, cytosolic free calcium concentration; $\Delta\psi_{mit}$, mitochondrial membrane potential; AM, acetomethoxy; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; BSA, bovine serum albumin; BCECF, 2',7'-bis((2-carboxyethyl)-5(6)-carboxyfluorescein; P/M, pyruvate + malate; F/O, carbonyl cyanide p-trifluoromethoxyphenylhydrazone + oligomycin; T/T/C, thapsigargin/di-tert-butyl hydroquinone/caffeine.

son *et al.*, 1989). Indeed, effective buffering of $[Ca^{2+}]_i$ is critical to neuronal survival (Scharfman and Schwartzkroin, 1989).

Intracellular Ca^{2+} regulation is known to be tightly linked to the energy status of the cell. In addition to gross energy failure which is associated with severe disturbances in Ca^{2+} levels and distribution, alterations in the ATP/ADP ratio provide a means for a more subtle modulation of $[Ca^{2+}]_i$ through their action on the various Ca^{2+} pumps and endoplasmic reticulum-type Ca^{2+} stores (Prentki *et al.*, 1984; Corkey *et al.*, 1988). Moreover, although yet an unexplored possibility, variations in energy availability in mitochondria (membrane potential or proton motive force) in otherwise healthy cells may also affect the capacity of Ca^{2+} accumulation in this organelle.

Understanding the trophic requirements of neuronal cells is an important step toward developing a therapeutical approach to neurodegeneration and improving *in vitro* cell culture. The aim of this study was to investigate whether the addition of pyruvate to neurons with unrestricted glucose supply would have effects on (*a*) the rate of respiration and ATP levels maintained by the cells and (*b*) the regulation of cell Ca^{2+} homeostasis. We have used a preparation of adult rat dissociated hippocampal and cerebrocortical cells (Villalba *et al.*, 1992) suitable for the determination of respiration rates, ATP levels, acetylcholine release, total and free Ca^{2+} levels, together with cultured hippocampal and cerebral cortex neurons, for $[Ca^{2+}]_i$ determination in single cells and $[Ca^{2+}]_i$ imaging. Our results indicate that the supply of pyruvate plus malate induces an alteration in cell Ca^{2+} regulation whereby total Ca^{2+} associated with the cells increases in the presence of lower $[Ca^{2+}]_i$. Under these conditions, both in cerebrocortical and especially hippocampal neurons, an FCCP-releasable Ca^{2+} pool becomes an important part of total intracellular Ca^{2+}. We have used $[Ca^{2+}]_i$ imaging in cultured neurons in combination with $^{45}Ca^{2+}$ labeling in brain cell suspensions to characterize the subcellular origin of FCCP-releasable Ca^{2+}. The partial resistance to external Ca^{2+} chelation and to maneuvers leading to ATP rundown suggests that the internal organelle harboring FCCP-released Ca^{2+} is the mitochondrion. The data are discussed in terms of the role of mitochondria in neuronal calcium handling.

MATERIALS AND METHODS

Preparation of Acutely Dissociated Rat Brain Cells—Acutely dissociated adult rat brain cells were obtained from hippocampus or cerebral cortex following a mild digestion with collagense in a medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.4 mM PO_4H_2K, 10 mM glucose, 10 mM Tris-HCl, pH 7.4 (medium A), or in that medium supplemented with pyruvate plus malate (2.5 mM each) (medium A + P/M) (Villalba *et al.*, 1992), and maintained at room temperature in the same medium until used. When used for oxygen uptake experiments, cells were suspended in 0.32 M sucrose, 10 mM Tris, 0.1 mM EDTA, 1% BSA and maintained on ice until used.

ATP and ADP Determinations—To measure ATP and ADP levels, dissociated hippocampal cells (0.5 mg and protein/ml) were incubated in medium A or medium A + P/M supplemented with 1 mM $CaCl_2$, for 15 min at 37 °C. ATP and ADP were extracted with 10% (w/v) trichloroacetic acid at 4 °C. After centrifugation, 10-μl aliquots of the supernatants were neutralized with 490 μl of 0.1 M Tris-acetate, 2 mM EDTA, pH 7.55, and ATP was determined with a luminescence test (Lemasters and Hackenbrock, 1978) as described elsewhere (Martínez-Serrano *et al.*, 1992). To evaluate ADP, 50-ml aliquots of the supernatants were neutralized with 900 ml of 0.1 M Tris-acetate, 2 mM $MgCl_2$, and 3 mM KCl and taken to pH 7.55 with NaOH. ADP was assayed after ATP, from the change in luminescence recorded after addition of 2.5 mM phosphocreatine (Sigma) and 2 units of creatine kinase (Boehringer Mannheim).

Acetylcholine Release—Acetylcholine release was estimated with the luminescence method modified for neutral pH (Gómez-Puertas *et al.*, 1991). Cells (0.8 mg of protein/ml) were preincubated for 15 min in the dark at 30 °C in medium A or medium A + P/M and 1 mM $CaCl_2$ in the presence of 10 units of choline oxidase, 11 units of peroxidase, 10 units of acetylcholinesterase, and 40 nmol of luminol in a final volume of 1 ml. Chemioluminescence was recorded in a LKB luminometer, and addi-

tions were made after decay of the luminescence signal. The endogenous content of acetylcholine was determined after trichloroacetic acid (20% w/v) extraction and processing of the samples (Willoughby, *et al.*, 1986). Aliquots (100 μl) were assayed with 100 μl of the chemioluminescence buffer mixture (see above) except that acetylcholinesterase was added in the luminometer. The signals obtained were calibrated with known doses of acetylcholine.

$^{45}Ca^{2+}$ Labeling Assays—$^{45}Ca^{2+}$ uptake in dissociated brain cells was studied essentially as previously described (Villalba *et al.*, 1992). Cells were preincubated in medium A or medium A + P/M for 5 min at 37 °C, and $^{45}Ca^{2+}$ accumulation was then initiated after addition of 1 mM $CaCl_2$, 1.5 μCi of $^{45}Ca^{2+}$/ml, and 29 nM ruthenium red (unless indicated otherwise) and continued for the times indicated in the figures. To study $^{45}Ca^{2+}$ uptake into free mitochondria, the mitochondrial preparations were obtained from the pellets after Ficoll gradient centrifugation of the crude synaptosomal fractions, P_2 (Vitórica, *et al.* 1984), suspended in 0.32 M sucrose, 10 mM Tris, pH 7.4, and stored on ice until used. $^{45}Ca^{2+}$ uptake was determined under the same conditions used for dissociated cells.

Determination of Total Ca^{2+}—Cells were incubated (2 mg/ml) in 1 mM $CaCl_2$, 29 nM ruthenium red in medium A + P/M or – P/M for 21 min. Then the medium was withdrawn by centrifugation, and cells were washed with 0.32 M sucrose, 10 mM Tris, 10 mM $LaCl_3$, pH 7.4, at 4C°. The cell pellets were then suspended in 1.5 ml of 2 N HCl in distilled water (Milli Q; Millipore), vortexed, centrifuged (15-min top speed bench centrifuge), and divided into three aliquots to which additions (1:1 by volume) of known amounts of ^{40}Ca (0, 1, and 2 ppm) were made. Samples were then mixed with equal volumes of a solution containing (w/w) 10% lanthanum, 20% HCl, 4% CsCl, and total Ca^{2+} was determined by atomic absorption spectroscopy.

Cytosolic pH—Cytosolic pH was measured with the fluorescent dye BCECF. Cells were loaded with 5 mM BCECF-AM (Molecular Probes) as described for fluo 3-AM and processed as indicated in Martínez-Serrano *et al.*, (1992).

$[Ca^{2+}]_i$ Determinations in Dissociated Brain Cells—For loading with fluo 3 or fura 2, cells were suspended in medium A (10 mg protein/ml) supplemented with 1% BSA, 5 μM (in dimethyl sulfoxide) fluo 3-AM or fura 2-AM (Molecular Probes) in the presence or absence of P/M. After 30-min incubation at 37 °C, cells were washed twice with medium A or medium A + P/M, 0.1% BSA, and maintained in the corresponding media at room temperature until used. For fluo 3 experiments excitation and emmission were at 506 and 526 nm, respectively. The corresponding wavelengths were 340 and 506 nm for fura 2. Any external free dye was eliminated by centrifugation of the cells (about 1 and 2 mg of protein for fluo 3 and fura 2, respectively) immediately before being used. To calibrate $[Ca^{2+}]_i$, F_{max} and F_{min} were obtained from the fluorescence observed after the addition of 160 μM digitonin plus 1 mM Cl_2Ca, and 10 mM EGTA plus 20 mM Tris, respectively. A K_D of 450 nM (fluo 3) or 224 nM (fura 2) was used. A detailed description of the fluorescence measurements is described in Villalba *et al.* (1992).

Preparation of Neuronal Cell Cultures—Primary cultures of neuronal cells from rat brain cortex were prepared essentially as described (Gomeza *et al.*, 1991). The cerebral cortex of 12–16 Wistar rat embryos, 17 days of gestation, were enzymatically dissociated in phosphate-buffered saline containing 1% BSA, 0.4 mg/ml papain, and 6 mM glucose. Dissociated cells were collected by centrifugation (800 × *g*, 5 min). Cell pellets were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. At this stage, more than 95% of the cells remained viable based on trypan blue exclusion. The cells were plated at 1.5 × 10⁵ cells/cm² on glass coverslips pretreated overnight with 10 μM poly-L-lysine and 2 h with laminin (4 μg/ml). The medium was replaced after 2 h by a serum-free medium consisting of Dulbecco's modified Eagle's medium and the components of B18 defined medium (Brewer and Cotman, 1989) (see Table I) omitting glutamate, glutamine, retinol, retinylacetate, catalase, superoxide dismutase, and triiodothyronine. This medium was changed again every 3 days. The experiments were performed after 6 days in culture.

$[Ca^{2+}]_i$ Microfluorimetry in Primary Neuronal Cultures—Cultured neurons growing on coverslips were washed twice in serum-free medium A and then incubated in the same medium or in medium A + P/M 4.5 h at 37 °C, changing the medium twice during this time. Neurons were then loaded with fura 2 by incubation in medium A or medium A + P/M supplemented with 0.1% BSA and 5 μM fura 2-AM for 30 min at 37 °C in a final volume of 1.5 ml. Following this incubation, the cells were washed twice in medum A or medium A + P/M, 0.1% BSA. No punctuate fluorescence was apparent in neurons loaded with this procedure, indicating that the dye was truly localized in the cytosol.

The coverslips containing the loaded and washed cells were mounted

in an open chamber (1 ml) and placed on the stage of a Zeiss Axiovert 35M inverted microscope. Fluorescence excitation was provided by a XB0 75-W xenon lamp through a dichroic mirror (Zeiss FT 395) and wavelengths (340 and 380 nm) were selected by a controlled (Zeiss MSP21 Microscope System Processor) movement of two filters (Zeiss BP 340 and 380) into the light path. The emmitted light (500–530) was collected by a Zeiss Plan-NEOFLUAR 40x/0.75 objective and measured by a photomultiplier (Zeiss MPM 200 Microscope Photometer). Exposure times were 12.5 ms and one I_{340}/I_{380} measurement was acquired in 2.5 s.

Fura 2 fluorescence from individual cells was measured at room temperature with a photomultiplier diaphragm of a diameter slightly larger than the cell body of the cell being measured (15 mm). Cells were washed four times with medium A or medium A + P/M by removing and replacing fresh medium on the stage of the microscope before recording fura 2 fluorescence. A base-line $[Ca^{2+}]_i$ was monitored in five selected cells from the same field, and then the $CaCl_2$ concentration of the medium was raised to 1 mM; the resulting fluorescence was again recorded in the same cells during the next 30 min, when a new steady state was obtained. Following subtraction of the background at both wavelengths, signal ratios were converted to $[Ca^{2+}]_i$ by using equation $[Ca^{2+}]_i = [(R - R_{min})/(R_{max} - R)]$. K_D (Grynkiewicz et al., 1985) R_{max}, R_{min}, and D were obtained from the fluorescence observed after addition of 160 μM digitonin (before dye leakeage) and 10 mM EGTA plus 10 mM Tris, respectively. A K_D of 224 nM was used. Background light levels (which included autofluorescence) were determined on a separate coverslip with cells that had not been loaded with fura 2. In a few experiments, a solution of fura 2 pentapotassium salt in medium A was added, and R_{max}, R_{min}, and D were also determined. R_{max} and R_{min} obtained this way did not differ significantly from those obtained as indicated above.

$[Ca^{2+}]_i$ Imaging—Cells were loaded with fura 2 by incubation in 2 ml of medium A or medium A + P/M and 2.5 μM fura 2-AM dissolved in dimethyl sulfoxide (final concentration, 1 μl of dimethyl sulfoxide/1 ml). After 50 min at 37 °C, fura 2-loaded cells were counterstained with rhodamine 123 (Sigma) by incubation with 10 μg/ml for 10 min prior being used. This medium was washed off by rinsing three times with medium A + P/M and 1 mM $CaCl_2$. The fura 2- and rhodamine 123-loaded cells were mounted in the microscope stage (Olympus inverted microscope, x40 Nikon oil immersion objective, numeric aperture = 1.3) for $[Ca^{2+}]_i$ imaging. The incubation chamber was thermostatically regulated at 37 ± 2 °C. A charge-coupled device intensified camera was used as the imaging device. Images at each wavelength were stored (an average of 32 images at each wavelength, one ratioed image, every 2 s) in an optical disk recorder or directly in the computer hard disk. Fura 2 was excited at 340 and 380 nm, respectively, and the emmitted light was filtered by a cut off at 460 nm. Rhodamine 123 was excited at 470 nm and the emmitted light filtered by a cut off at 490 nm.

Background fluorescence of fura 2-loaded cells was estimated after 20-min incubation of the cells in the presence of 1 mM $MnCl_2$. Under these conditions, fluorescence of the cells was not changed by ionomycin, indicating a complete quenching of the dye by manganese and suggesting that the dye was exclusively cytosolic. Data were analyzed by transfer of images from the optical disk recorder to an image processing system (Georgia Instruments Inc). Fluorescence ratios (340/380) were determined after substraction of background fluorescence. No attempt was made of converting 340/380 ratios to actual $[Ca^{2+}]_i$ values.

Neurons were continuously superfused with incubation medium. A pumping device operated the inlet tubing and the outlet was connected to a vacuum supply, allowing a two-way regulation of the chamber volume. Media were changed under continuous superfusion unless hydrophobic compounds were used. In order to add these compounds (FCCP, oligomycin, antimycin), the volume of the incubation media was first reduced to 0.5 ml, pumping was then interrupted, and 0.5 ml of incubation media containing appropriate FCCP and oligomycin concentrations were added right under the inlet of the superfusion tubing. Pumping was immediately resumed and again stopped when the incubation volume reached 0.5 ml. At the concentrations used, none of these compounds interfered with fura 2 fluorescence. The temperature of the chamber did not change during interruption of superfusion.

RESULTS

Effect of Exogenous Pyruvate on Respiration and ATP Levels—In synaptic terminals, glucose oxidation via pyruvate dehydrogenase requires the presence of a source of oxalacetate which may be provided by malate or aspartate *in vivo* (Cheesman and Clark, 1988). Since we were interested in understand-
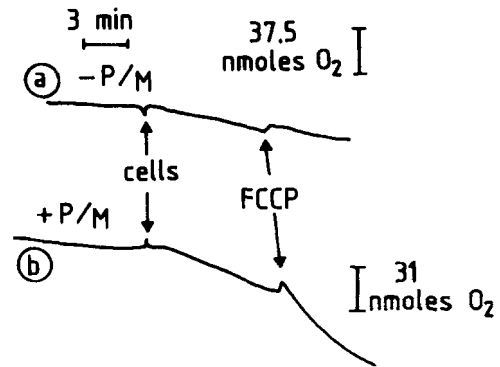

FIG. 1. **Effect of exogenous respiratory substrates on respiration of dissociated brain cells.** Tracings were obtained with a Clark type oxygen electrode. Measurements were carried out in medium A supplemented with 2 mg BSA/ml and 1 mM $CaCl_2$ at 30 °C in the absence (*a*) or presence (*b*) of 2.5 mM pyruvate + 2.5 mM malate (1.7 ml final volume) with gentle magnetic stirring. Where indicated (*cells*), dissociated cortical cells (0.41 mg of protein/ml in (*a*) or 0.47 mg of protein/ml in (*b*) were added. *FCCP* (10 μM) addition is also indicated.

ing the trophic effects of pyruvate in terms of energy supply, we have used a combination of pyruvate and malate in order to optimize pyruvate oxidation. No attempt was made at analyzing the effects of pyruvate or malate separately.

Fig. 1 shows the effects of the addition of exogenous P/M on the respiration of brain cell suspensions incubated in the presence of 10 mM glucose. It may be observed that the respiration rate was significantly increased (2.94 ± 0.70 or 5.03 ± 0.78 nmol of O_2 × mg of protein^{-1} × min^{-1} in medium A or medium A + P/M, respectively, mean ± S.E. of six experiments $p < 0.05$). However, the ATP content of the cells was not altered (8.3 ± 0.4 or 8.0 ± 0.6 nmol of ATP × mg of protein^{-1} in the absence or presence of P/M, respectively).

Effect of Exogenous Pyruvate + Malate on $[Ca^{2+}]_i$ Regulation in Dissociated Brain Cells and Cultured Neurons—The effects of exogenous P/M on $[Ca^{2+}]_i$ regulation were studied in fluo 3-loaded dissociated hippocampal and cerebral cortex (not shown) dissociated cells. The $[Ca^{2+}]_i$ levels maintained at rest by these brain cells are significantly lower when P/M was present in incubation medium (Table I).

Our dissociated brain cell preparations are made up of neurons and glial cells together with other cell types. The proportion of neurons was 69% and 72% in hippocampal and cerebral cortex preparations, respectively (Villalba *et al.*, 1992). To study the effects of the addition of exogenous respiratory substrates on a purely neuronal population, we cultured astrocyte-free cortical neurons and analyzed the effects of exogenous substrates on the $[Ca^{2+}]_i$ maintained by these cells. The variations in $[Ca^{2+}]_i$ in single cells under a fluorescence microscope were studied with fura 2. Table I shows that addition of P/M produced similar effects as those observed in dissociated brain cells: $[Ca^{2+}]_i$ levels in resting cells were markedly lower in neurons supplied with exogenous P/M than in those incubated with glucose alone. In fact, the presence of P/M had a more pronounced effect in this purely neuronal preparation than in mixed dissociated brain cells (Table I).

Effect of Exogenous Pyruvate + Malate on Total Ca^{2+} Levels—Total Ca^{2+} levels in dissociated brain cells incubated in the presence or absence of exogenous P/M were determined after labeling the cellular Ca^{2+} pools with $^{45}Ca^{2+}$. Fig. 2 shows that P/M addition led to an increase in total $^{45}Ca^{2+}$ accumulation of about 2–3 nmol/mg of protein in hippocampal cells (and cerebral cortex cells, results not shown) after 20 min, a time at which $^{45}Ca^{2+}$ loading in medium A + P/M leveled off. This increase disappeared at later times. The difference in $^{45}Ca^{2+}$ loading observed at 20 min is not merely a consequence of a

TABLE I

Effect of the presence of exogenous pyruvate + malate (2.5 mM each) on resting $[Ca^{2+}]_i$ and on F/O- or A/O-induced increases in $[Ca^{2+}]_i$ in dissociated hippocampal cells and cultured cerebral cortex neurons

Fluo 3-loaded hippocampal cells (1 mg of protein) were preincubated for 20 min at 37 °C in medium A supplemented with 1 mM $CaCl_2$ and with (+P/M) or without (−P/M) pyruvate + malate (2.5 mM each) before starting $[Ca_{2+}]_i$ determinations. When a steady state $[Ca^{2+}]_i$ level was observed, 10 µM FCCP + 10 µM oligomycin (F/O), or 10 µM antimycin + 10 µM oligomycin (A/O) were added. Data represent mean ± S.E. values (three–six experiments performed in triplicate) of $[Ca^{2+}]_i$ values observed at rest and 90 s after F/O or A/O (10 µM each) addition. The difference between those values and resting $[Ca^{2+}]_i$ is indicated as $\Delta[Ca^{2+}]_i$. $[Ca^{2+}]_i$ determinations in cultured neurons were carried out as described under "Materials and Methods." The calcium concentration of medium A (measured with fura 2) was about 1 µM. Data were obtained after stable $[Ca^{2+}]_i$ levels were recorded and represent mean ± S.E. in 60 single cells (four experiments performed in triplicate).

Conditions	Additions	Dissociated hippocampal cells		Cultured cortical neurons $[Ca^{2+}]_i$	
		$[Ca^{2+}]_i$	$\Delta[Ca^{2+}]_i$	$[Ca^{2+}]_o$, 1 µM	$[Ca^{2+}]_o$, 1 mM
		nM		*nM*	
−P/M		283 ± 5			
+P/M		243 ± 2***[a]			
−P/M	F/O	413 ± 4	138 ± 6		
+P/M	F/O	428 ± 4	180 ± 4***		
−P/M	A/O	308 ± 10	25 ± 6		
+P/M	A/O	304 ± 5	61 ± 6**		
−P/M				27.7 ± 1.2	149.8 ± 15.5
+P/M				18.7 ± 1.7*	95.1 ± 9.5*

[a] The significance of the difference between values with or without P/M was (student's *t* test) * $p < 0.025$; ** $p < 0.005$; *** $p < 0.0005$.

different labeling rate, but reflects a real difference in total Ca^{2+} associated with the cells. Thus, total Ca^{2+} levels determined by atomic absorption spectroscopy were 14.70 ± 2.79 or 27.55 ± 3.3 nmol/mg of protein in hippocampal cells in the absence or presence of P/M, respectively ($p < 0.01$), and 13.20 ± 1.85 *versus* 27.81 ± 7.38 nmol/mg of protein in cortical cells with or without P/M, respectively ($p < 0.05$). The larger size of total ^{40}Ca with respect to the $^{45}Ca^{2+}$-labeled pool indicates the presence of the slowly exchanging Ca^{2+} pool(s) that remains unlabeled during the loading period. Taken together, the results indicate that the provision of exogenous P/M increases both rapidly exchangeable and slowly exchangeable Ca^{2+} pools.

Contribution of Different Ca^{2+} Stores to Increased Ca^{2+} Accumulation in the Presence of Pyruvate + Malate—Rapidly exchangeable, agonist-sensitive Ca^{2+} stores are known to represent one of the Ca^{2+} pools present in various cell types (Fasolato *et al.*, 1991; Meldolesi *et al.*, 1990; Rossier and Putney, 1991). The contribution of these Ca^{2+} stores to total Ca^{2+} accumulation may be evaluated from the $^{45}Ca^{2+}$ released from the cells after the addition of the Ca-ATPase inhibitors thapsigargin and 2,5-di-*tert*-butyl-1,4-hydroquinone (Thastrup *et al.*, 1990; Moore *et al.*, 1987), together with caffeine that releases Ca^{2+} from stores functionally distinct from those sensitive to inositol 1,4,5-triphosphate (Law *et al.*, 1990) (T/T/C, thapsigargin/di-*tert*-butyl hydroquinone/caffeine).

Mitochondria are also known to account for an important fraction of total Ca^{2+} accumulation in these structures (Akerman and Nicholls, 1981; Vitórica and Satrústegui, 1986; Martínez-Serrano and Satrústegui 1992), and recent reports suggest that they may also participate actively in calcium homeostasis in different neuronal types (Miller, 1992; Duchen, 1992). In the following experiments we have tried to determine the contribution of mitochondria to the rapidly exchangeable Ca^{2+} pool. To this end, we have measured the amount of $^{45}Ca^{2+}$ released from the cells after addition of the uncoupler FCCP.

Oligomycin was added together with the uncoupler. (Alternatively, a respiratory chain inhibitor, 10 µM antimycin, was used instead of FCCP.) The rationale is that the collapse of the mitochondrial membrane potential, $\Delta\psi_{mit}$, by FCCP + oligomycin or antimycin + oligomycin should lead to a specific and rapid release of mitochondrial Ca^{2+} into the cytoplasm, followed by its extrusion into the medium.

Fig. 3 shows the effect of exogenous substrates on the T/T/C and FCCP/oligomycin (F/O) Ca^{2+} pools. The T/T/C pool in cortical cells (Fig. 3A) represented around 10–15% of total $^{45}Ca^{2+}$, increasing from below 1 nmol/mg of protein in the presence of glucose alone to over 1.5 nmol/mg of protein in A + P/M (Fig. 3A). No T/T/C $^{45}Ca^{2+}$ pool was detected in hippocampal cells (Fig. 3B). Fig. 3B shows that in the presence of glucose alone, the fraction of F/O-releaseable Ca^{2+} was relatively small (within 15% of total $^{45}Ca^{2+}$) in hippocampal cells, whereas the addition of P/M increased the fraction of F/O-releaseable $^{45}Ca^{2+}$ by 2-fold. The effect of P/M on the F/O Ca^{2+} pool was less marked in cortical cells (Fig. 3A). These results indicate that the presence of exogenous substrates increases total Ca^{2+} accumulation in brain cells by increasing F/O and probably T/T/C Ca^{2+} pools, the relative effects on both of these pools depending on the cell type. Therefore, in spite of unchanged ATP levels, the increased energy available leads to increased Ca^{2+} accumulation in endoplasmic reticulum-type and especially F/O Ca^{2+} stores.

It is unlikely that the Ca^{2+} released by F/O is redistributed into any other intracellular ATP-dependent Ca^{2+} pool after uncoupler addition since there is a substantial ATP drop under these conditions (see below and results not shown). However, it could be argued that T/T/C-mobilized Ca^{2+} might redistribute into the F/O pool, leading to an underestimation of the former during the $^{45}Ca^{2+}$ release experiments. Indeed, these two Ca^{2+} pools did not appear to be completely independent; thus, in cortical cells the F/O pool did not change by previous depletion of the T/T/C pool, but the simultaneous addition of T/T/C and F/O resulted in a $^{45}Ca^{2+}$ release which was larger than that obtained after their sequential addition (results not shown). These results suggest the $^{45}Ca^{2+}$ release protocol may underestimate the T/T/C Ca^{2+} pool.

In the experiments presented so far we have assumed that $^{45}Ca^{2+}$ accumulation and release from F/O stores represents a true intracellular Ca^{2+} pool. However, it may be argued that F/O stores represent contaminating free mitochondria or mitochondria in damaged cells. The results depicted in Fig. 4 exclude this possibility. Thus, 29 nM ruthenium red, a concentration which completely blocks Ca^{2+} uptake in isolated mitochondria incubated under the conditions used for cell suspensions (results not shown), had no effect on cell $^{45}Ca^{2+}$ accumulation or on the amount of F/O-releaseable $^{45}Ca^{2+}$ irrespective of the presence or absence of exogenous P/M, except that the time course of $^{45}Ca^{2+}$ loss after F/O addition was somewhat slower (Fig. 4). This indicates that the increase in $^{45}Ca^{2+}$ content of dissociated brain cells incubated with exogenous respiratory substrates is not attributable to a few damaged cells, but rather suggests that the effects of P/M are related to the existence of specific plasma membrane transport systems for these metabolites (Shank and Campbell, 1984). It should be noted that the ruthenium red concentrations used in these experiments (29 nM) are about 2 orders of magnitude lower than those causing an inhibitory effect on Ca^{2+} currents in single dissociated mouse sensory neurons (Duchen, 1992).

Effect of Exogenous Pyruvate + Malate on F/O and T/T/C $[Ca^{2+}]_i$ Responses and Acetylcholine Release in Dissociated Brain Cells—To study the effects of F/O on $[Ca^{2+}]_i$ in dissociated brain cells we have utilized fluo 3 to avoid artifacts due to FCCP fluorescence and because fluo 3 fluorescence is independ-

FIG. 2. Effect of exogenous respiratory substrates on $^{45}Ca^{2+}$ accumulation in dissociated brain cells. Hippocampal cells were preincubated in medium A in the presence (O) or absence (●) of pyruvate + malate (2.5 mM each) for 5 min at 37 °C. $^{45}Ca^{2+}$ accumulation was then initiated and continued for 60 min at 37 °C (O, ●). Data are mean ± S.E. of three experiments performed in triplicate. The significance of the difference between the values with or without P/M was (Student's *t* test): *$p < 0.05$.
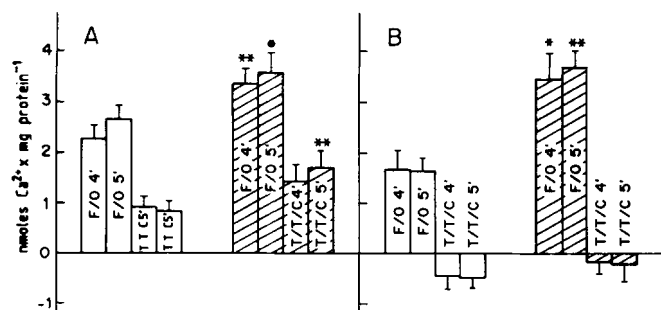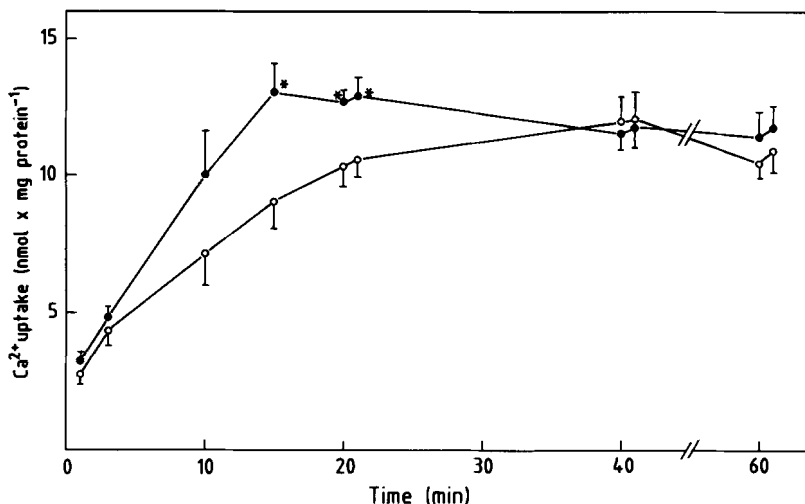

FIG. 3. Effect of exogenous pyruvate + malate on the $^{45}Ca^{2+}$ pools mobilized by caffeine + thapsigargin + *tert*-butyl hydroquinone and FCCP/oligomycin. Brain cells derived from cerebral cortex (*A*) or hippocampus (*B*) were labeled with $^{45}Ca^{2+}$ during a 21-min incubation in basal media in the presence of glucose and with (*striped bars*) or without (*open bars*) exogenous P/M. Then, a mixture of 10 mM caffeine, 1 µM thapsigargin (Sigma), and 25 µM *tert*-butyl hydroquinone (Aldrich) (*T/T/C*) or FCCP/oligomycin (*F/O*) (10 µM each) was added, and 4 or 5 min later, the incubation was stopped and $^{45}Ca^{2+}$ accumulation was determined in cells treated with T/T/C, F/O, or no additions. The loss of $^{45}Ca^{2+}$ from the cells either after F/O or T/T/C addition was the same at 4 and 5 min, indicating that the release of $^{45}Ca^{2+}$ was largely completed. F/O- or T/T/C-released $^{45}Ca^{2+}$ (at 4 or 5 min) represents the difference in $^{45}Ca^{2+}$ accumulation between control and F/O- or T/T/C-treated cells (mean ± S.E. of four to eight experiments). The significance of the difference between the values with or without P/M was (Student's *t* test): *$p < 0.05$; **$p < 0.025$.

ent of cellular pH over a wide pH range (Minta *et al.*, 1989) and it does not accumulate in synaptosomal mitochondria (Martínez-Serrano and Satrústegui, 1992). The collapse of the $\Delta\psi_{mit}$ by F/O leads to a rise in $[Ca^{2+}]_i$ which was larger when P/M was exogenously supplied (Fig. 5A and Table I). A similar, albeit slower, increase was obtained with antimycin + oligomycin (Fig. 5B, Table I).

To test the possibility that the increase in F/O-releasable Ca^{2+} pool (Figs. 3, 4, and 5A and Table I) obtained by supplying exogenous P/M could also result in an increase in neurotransmitter release, we studied the release of acetylcholine obtained after F/O (or antimycin/oligomycin, not shown) addition to dissociated brain cells. Fig. 5C shows that acetylcholine release was significantly increased when the respiratory substrates were supplied exogenously. This was not due to changes in endogenous acetylcholine content, since this was unchanged after incubation with P/M. In fact, the congruency obtained between these results and those of Ca^{2+} mobilization (Fig. 5A, Table I) was impressive.

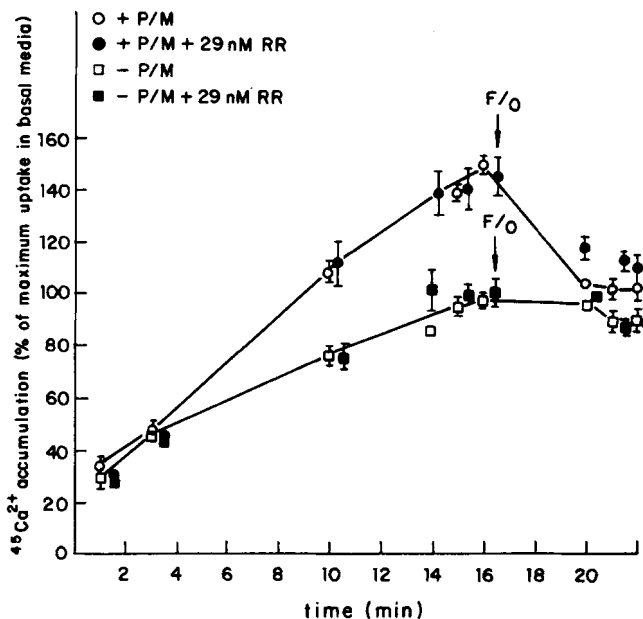The effects of T/T/C on $[Ca^{2+}]_i$ were determined separately in

FIG. 4. Effects of ruthenium red on $^{45}Ca^{2+}$ accumulation in dissociated hippocampal cells. Calcium uptake in dissociated cells incubated in the presence (O, ●) or absence (□, ■) of P/M. At 16 min, 10 µM FCCP + 10 µM oligomycin (*F/O*) were added. Closed symbols correspond to incubations in the presence of 29 nM ruthenium red which was added during preincubation. The results are expressed as percentage of the maximum uptake observed in medium A. Data are mean ± S.E. of three experiments performed in duplicate.

fura 2- (caffeine) and fluo 3- (thapsigargin and *tert*-butyl hydroquinone) loaded cells, since caffeine interferes with fluo 3. The response to caffeine was (increase in $[Ca^{2+}]_i$ measured at 90 s after drug addition, mean ± S.E. of 4–10 determinations): 129 ± 2 and 146 ± 11 nM in cerebral cortex and hippocampus, respectively, while those to thapsigargin (33 ± 4 and 40 ± 3 nM) or *tert*-butyl hydroquinone (17 ± 3 and 18 ± 3 nM) were much smaller and did not vary in the absence or presence of P/M (results not shown). Since T/T/C effects on $[Ca^{2+}]_i$ were similar in cerebral cortex and hippocampal cells, whereas no T/T/C $^{45}Ca^{2+}$ pool was detected in hippocampus (Fig. 3), these results further emphasize the point that there is calcium redistribution among intracellular calcium stores after T/T/C addition (see above).

Characterization of FCCP Effects on $[Ca^{2+}]_i$ in Cultured Neurons—The results presented so far indicate that the F/O-induced $[Ca^{2+}]_i$ rise in dissociated brain cells increases in the

A

B

C
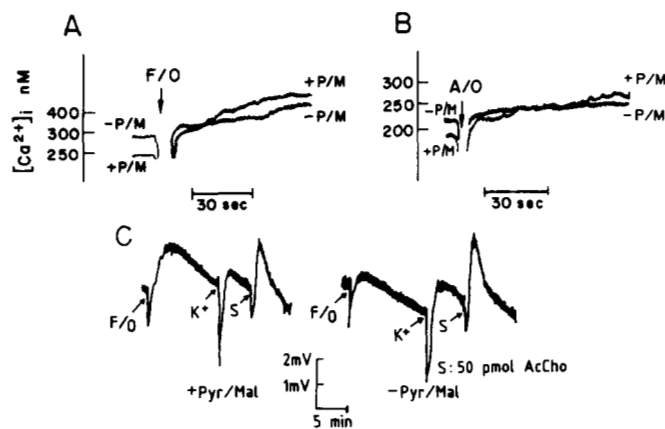
FIG. 5. **Effect of exogenous P/M on F/O-induced increases in** [Ca^{2+}]$_i$ **and acetylcholine release.** A and b, fluo 3-loaded hippocampal cells (1 mg of protein) were preincubated for 20 min at 37 °C in medium A supplemented with 1 mM CaCl$_2$ and with (+P/M) or without (-P/M) pyruvate + malate (2.5 mM each), and [Ca^{2+}]$_i$ determinations were then initiated. Where indicated, 10 µM FCCP + 10 µM oligomycin (F/O), or 10 µM antimycin + 10 µM oligomycin (A/O) were added. Results are representative fluorescence tracings. C, luminescence recordings of acetylcholine release in hippocampal cells preincubated in the absence or presence of 2.5 mM pyruvate + 2.5 mM malate. After decay of the luminescence signal, FCCP + oligomycin (10 µM each) were added at the *arrow*. The signal obtained after the addition of known doses of acetylcholine is also shown. F/O-induced acetylcholine release was 62.5 ± 3.2 pmol/mg of protein (+Pyr/Mal) or 41 ± 2.1 pmol/mg of protein (-Pyr/Mal) (mean ± S.E. of three experiments; the significance of the difference was $p < 0.005$). Endogenous acetylcholine was 121 ± 10.7 (+Pyr/Mal) and 118.4 ± 15.9 (-Pyr/Mal) pmol/mg of protein. The same results were obtained when 5 µM antimycin was used instead of FCCP.

presence of P/M. To study the source of FCCP-released Ca^{2+}, we next characterized the F/O-induced [Ca^{2+}]$_i$ increases in cultured neurons. To this end, we studied the response to F/O in cultured hippocampal neurons (and cerebrocortical neurons, not shown) by Ca^{2+} imaging. The effect of F/O on [Ca^{2+}]$_i$ consists of an increase in cytosolic calcium which varies in size among the cells. In some cells it consists of an initial peak which is followed by a decay to base line, whereas in others the increase is gradual and sustained. A similar response pattern was observed when 2 µM antimycin was used instead of FCCP (results not shown).

Addition of EGTA 6–30 s before F/O (final EGTA and Ca^{2+} concentrations, 2.5 and 0.5 mM, respectively), a condition under which a K$^+$-induced [Ca^{2+}]$_i$ rise was completely blocked (Fig. 6B) (indicating that Ca^{2+} influx was abolished), usually resulted in a smaller response (Fig. 6, compare *Panels A* and *B*). The reduction of F/O responses by EGTA may be due to a rapid depletion of the F/O-releasable Ca^{2+} pool through equilibration with cytosolic and external Ca^{2+}. We have tested this possibility by comparing the sizes of the F/O released ^{45}Ca^{2+} pools in dissociated cerebral cortex cells in the presence or absence of EGTA (final EGTA and Ca^{2+} concentrations, 4 and 1 mM, respectively) added 6 s before F/O. The F/O-released ^{45}Ca^{2+} pool was somewhat smaller (1.21 ± 0.08 and 0.86 ± 0.06 nmol/mg of protein, in Ca^{2+} or EGTA medium, respectively; $p < 0.01$) in the presence of EGTA and remained constant 1–4 min after F/O addition (results not shown). These results suggest that the reduction of the F/O-induced [Ca^{2+}]$_i$ rise by EGTA may be due in part to a rapid discharge of the F/O Ca^{2+} pool. However, the decrease in [Ca^{2+}]$_i$ rise is larger than that of the F/O-released ^{45}Ca^{2+}, indicating that another part of the [Ca^{2+}]$_i$ rise induced by mitochondrial inhibitors may be due to Ca^{2+} entry.

It could be argued that F/O addition causes ATP depletion and thereby a reduction of ATP-driven Ca^{2+} efflux across the plasma membrane or sequestration by intracellular organelles

other than mitochondria. It has been reported that FCCP releases Ca^{2+} from a non-mitochondrial store in *Helisoma* and bullfrog sympathetic neurons (Jensen and Reher, 1991; Marrion and Adams, 1992). To evaluate the effect of ATP depletion *per se*, cells were incubated in a glucose-free medium (where pyruvate and malate were also ommitted), and deoxyglucose was supplied in order to inhibit endogenous glycogen utilization. Oligomycin was then added to block mitochondrial ATP production without affecting the Δψ$_{mit}$. In these experiments cells were doubly loaded with fura 2 (to measure [Ca^{2+}]$_i$) and rhodamine 123 (to estimate Δψ$_{mit}$) (Chen, 1988; Chacon *et al.*, 1992).

To estimate the changes in ATP and ADP levels in these experiments, adenine nucleotides were evaluated in dissociated hippocampal cells incubated under the same conditions as neuronal cultures. Fig. 6F shows that the ATP/ADP ratio dropped slightly by incubation in a glucose-free medium whether or not exogenous P/M was supplied. ATP/ADP decreased markedly in the presence of deoxyglucose when the cells were not supplied with P/M, but the ATP/ADP ratio was stable in its presence. The addition of oligomycin resulted in a very rapid ATP/ADP drop to below 0.5, 2 (without P/M) or 5 (with P/M) min later. No further changes in ATP/ADP were observed after FCCP addition (Fig. 6F).

Fig. 6, C–E, shows that 12-min perfusion in the presence of 2 mM deoxyglucose in a glucose-free medium did not result in any immediate change in cytosolic calcium (14 out of 16 cells). A slight increase in 348/380 was observed in some of the cells after 10 min of incubation under these conditions (see Fig. 6, D and E). Basal 340/380 ratio was 0.82 ± 0.03 and 1.02 ± 0.04 after 10 min of incubation with 2 mM deoxyglucose (34 cells, mean ± S.E.). Addition of oligomycin resulted in a slow gradual increase in [Ca^{2+}]$_i$ (20 out of 20 cells), which in some cells started immediately (Fig. 6E) while in others was visible only after a prolonged lag (Fig. 6C). The [Ca^{2+}]$_i$ increase was modest in size: the 340/380 ratio increased from 1.02 ± 0.04 to 1.44 ± 0.06 (34 cells, mean ± S.E.) 10 min after oligomycin addition. There was no appreciable change in Δψ$_{mit}$ during deoxyglucose or oligomycin treatment as indicated by rhodamine 123 fluorescence intensity (results not shown). Addition of FCCP to these cells resulted now in an increase in 340/380 ratio, which was again variable from cell to cell (Fig. 6). It should be noticed that the size of the F/O peak is smaller than in naive cells in this type of experiment; thus, the mean 340/380 ratio after F/O was 2.59 ± 0.38 (20 cells) in naive cells and 2.42 ± 0.17 (34 cells) in those subjected to the deoxyglucose/oligomycin protocol; the increase obtained by FCCP itself being from 1.44 to 2.42. This may be due to a partial discharge of the F/O pool before FCCP addition.

Taken together, these results highlight the fact that [Ca^{2+}]$_i$ levels in hippocampal neurons increase little and slowly even after a drastic reduction in ATP. A similar situation has been described in hepatocytes (Brecht *et al.*, 1992). In contrast with this, [Ca^{2+}]$_i$ regulation in type I cells from the carotid body is more sensitive to ATP depletion since oligomycin addition results in a large rise in [Ca^{2+}]$_i$ (Duchen and Biscoe, 1992). On the other hand, these results clearly indicate that the F/O-induced [Ca^{2+}]$_i$ rise does not occur from a drop in the ATP/ADP ratio.

DISCUSSION

Effects of Exogenous Pyruvate on Neuronal Ca^{2+} Homeostasis—The requirement for pyruvate by cultured CNS neurons and their inability to survive when glucose is the sole energy source imply that neuronal production of endogenous pyruvate from glucose is insufficient to meet the neuronal demands, or it is overmatched by pyruvate losses (Facci et al. 1985). The fact

FIG. 6. **Effect of FCCP, deoxyglucose, and oligomycin on [Ca²⁺]ᵢ and ATP levels.** *A* and *B*, effects of external calcium chelation on F/O responses. Cultured hippocampal neurons were superfused with medium A + P/M (final Ca²⁺, 1 mM) (0.5 ml/min), and where indicated, the incubation medium was switched to high potassium (*65K*, 65 mM KCl), EGTA (2.5 mM EGTA and 0.5 mM CaCl₂), or mid potassium medium (*20K*, 20 mM KCl; *15K*, 15 mM KCl). F/O (3 μM FCCP, 10 μM oligomycin) was added as a bolus where indicated. Note that recordings were interrupted for 5–10 min as indicated (//) and superfusion with medium A + P/M, 1 mM CaCl₂ was resumed after F/O addition. *C–E*, cultured hippocampal neurons were superfused in glucose-free medium A (minus pyruvate and malate) and 1 mM CaCl₂. Twenty minutes later, 2 mM 2-deoxyglucose was added, and superfusion was continued for 12 min. Oligomycin (10 μM) was then added, and FCCP (2 μM) was added 10 min later, where indicated. *C–E* correspond to fura 2 340/380 fluorescence recordings in three representative cells. Note that recording was interrupted twice for 3 and 5 min where indicated (* and *', respectively). *F*, acutely dissociated hippocampal cells incubated in the absence (○) or presence (●) of 2.5 mM pyruvate + 2.5 mM malate were suspended in glucose-free medium for 20 min, and the different additions were carried out as indicated. ATP and ADP contents were determined as described under "Materials and Methods."
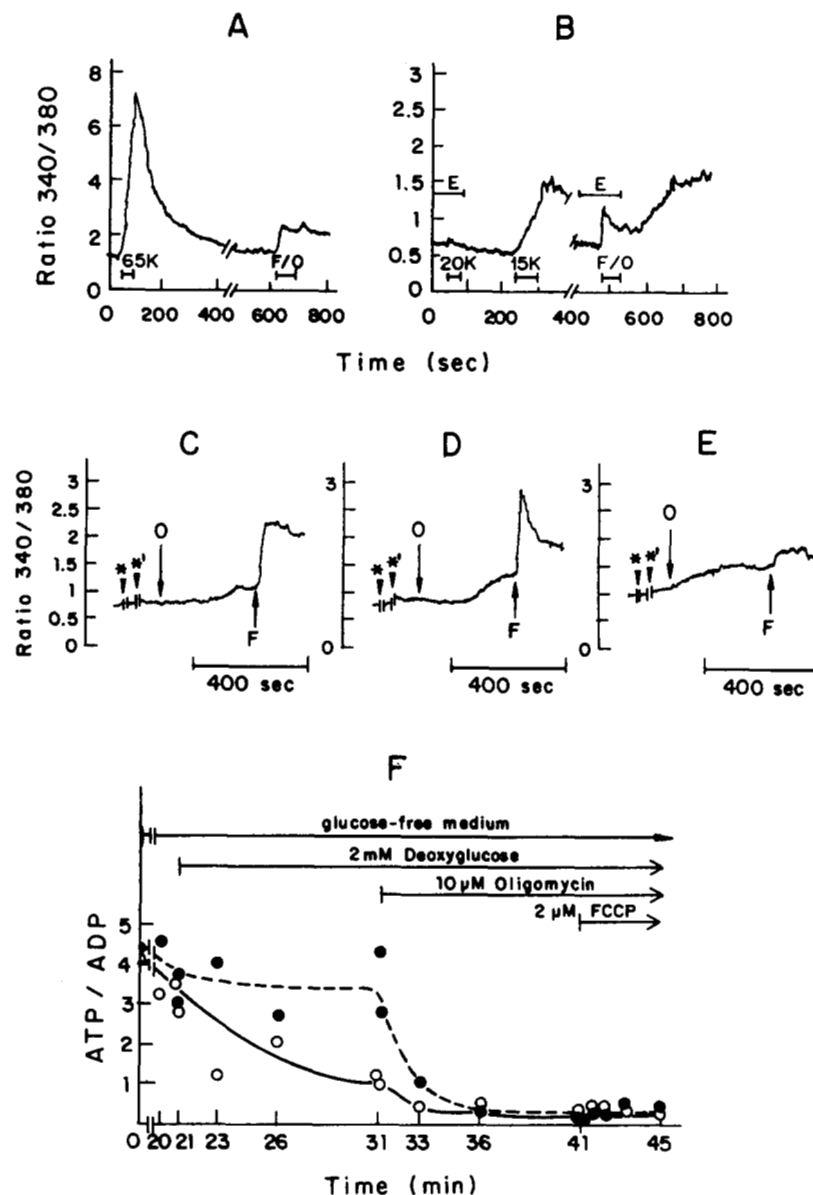
that exogenous pyruvate plus malate increases the respiration rate of dissociated CNS neurons (Fig. 1) indicates that these substrates fuel directly or indirectly the tricarboxylic acid cycle under conditions of unrestricted glucose supply.

The increase in the respiratory rate was not associated with changes in ATP levels. This was not entirely unexpected, since it is known that in other cell types (hepatocytes), incubated under resting conditions, a significant proportion (20–40%) of O₂ consumption in the presence of glucose alone may be due to H⁺ cycling across the inner mitochondrial membrane rather than ATP synthesis. Moreover, the addition of exogenous respiratory substrates is known to raise the $\Delta\psi_{mit}$ and results in an increase in the respiratory rate (Nobes *et al.*, 1990a, 1990b).

We have found that the the supply of P/M to acutely dissociated CNS cells results in a long lasting reduction of [Ca²⁺]ᵢ while total cell Ca²⁺ levels increases (Table I, Fig. 2). These changes are probably attributable to the neurons rather than to the other cells (*e.g.* glial cells) present in the mixed populations since they were more pronounced in pure neuronal cultures. Taken together, these results suggest that the exogenous supply of Krebs cycle substrates leads to an improved function of Ca²⁺ homeostatic mechanisms, in particular, increasing the

efficiency of Ca²⁺ extrusion into the medium (lower [Ca²⁺]ᵢ in the presence of P/M) and of Ca²⁺ accumulation in the intracellular stores (higher total Ca²⁺ content).

The improved capacity of extruding and sequestering Ca²⁺ conferred to the neurons by P/M might be of utmost practical importance. In fact, responses to external stimuli in terms of growth or cytosolic calcium signaling vary among neuronal types in a cell- and state-specific manner (Holliday *et al.*, 1991; Reber and Reuter, 1991). Milles and Kater (1990) have shown that the Ca²⁺ buffering capacity is one of the factors conferring cell and growth state specificity in cultured *Helisoma* neurons. Strong Ca²⁺ buffering of external Ca²⁺ loads was associated with resistance to cell death mediated by high doses of the Ca²⁺ ionophore A23187. The Ca²⁺ buffering capacity of presynaptic endings is unusually large and diminishes as a consequence of ageing (Martínez-Serrano *et al.*, 1992). Since effective buffering of intracellular Ca²⁺ during periods of neuronal excitation is crucial to cell survival (Scharfman and Schwartzkroin, 1989) the administration of exogenous pyruvate and malate could be of a therapeutic use to protect against non-ischemic events of high level activation of synaptic connections.

Effects of Exogenous Pyruvate plus Malate on Intracellular

Ca²⁺ Pools—As mentioned above, treatment of neurons with P/M results in an increase in the total cell Ca^{2+} content. In particular, the supply of P/M increases the size of the FCCP/oligomycin-releasable Ca^{2+} pool in hippocampal and cerebral cortex cells and that of the TTC-sensitive Ca^{2+} pool at least in cerebrocortical cells. The effects of P/M on the TTC-sensitive Ca^{2+} pool may be larger than observed in this study since our $^{45}Ca^{2+}$ release protocol probably underestimates the size of this pool. The effect of P/M supply caused both an increase of the rate of isotopic labeling of these pools (results not shown) and of their absolute size. Whether the effects of P/M on these pools is transient could not be determined because the large elevation of $[Ca^{2+}]_i$ in cells not supplied with P/M during prolonged incubation (results not shown) does not permit unambiguous conclusions to solve this problem.

The definition of "TTC-sensitive Ca^{2+} pool" reflects a functional property of this store, but its morphological identity remains to be established. On the contrary, the present results strongly suggest that the F/O-releasable Ca^{2+} pool corresponds to mitochondria. The following lines of evidence support this conclusion. (i) The F/O-induced $[Ca^{2+}]_i$ rise in resting CNS cultured neurons supplied with exogenous P/M is at least in part of intracellular origin since blocking Ca^{2+} influx across the plasma membrane by external EGTA or nickel (not shown) reduces, but does not abolish the F/O-induced $[Ca^{2+}]_i$ rise (Fig. 6). (ii) F/O addition to dissociated brain cells leads to a net cell $^{45}Ca^{2+}$ loss even in the presence of external Ca^{2+}. (iii) The slow increase of $[Ca^{2+}]_i$ in the face of maneuvers depleting cellular ATP to levels comparable to those obtained with F/O (Fig. 6) indicates that the effects or F/O are only in part due to the drop in the ATP/ADP ratio. (iv) A rapid increase in $[Ca^{2+}]_i$ is also produced by antimycin/oligomycin, and even when the ATP/ADP ratio has been previously reduced (Fig. 6). In conclusion, these results indicate that in neurons (incubated with P/M) mitochondria trap a relatively large amount of Ca^{2+} which is rapidly released into the cytosol when their $\Delta\psi_{mit}$ is collapsed. Treatment with F/O results also in an increased Ca^{2+} influx, whose mechanism, however, remains to be established.

We do not know at present what is the actual mechanism of P/M in promoting Ca^{2+} accumulation in mitochondria. One possibility is that the increase in the respiratory rate in the presence of P/M results in an increased $\Delta\psi_{mit}$. Kauppinen and Nicholls (1986) have in fact shown that the presence of exogenous pyruvate in synaptosomes increases $\Delta\psi_{mit}$. Wingrove *et al.* (1984) have demonstrated that Ca^{2+} influx in isolated mitochondria depends on $\Delta\psi_{mit}$ also at high membrane potentials (see Gunter and Pfeiffer (1990) for review). An alternative possibility is that P/M is needed to support the production of a yet unknown factor(s) which increases the Ca^{2+} affinity of the uptake process. It is important to stress that the accumulation of Ca^{2+} in mitochondria *in situ* does not lead to uncoupling of oxidative phosphorylation, since respiration was further enhanced by FCCP addition (Fig. 1). In fact, the presence of P/M leads both to a larger Ca^{2+} accumulation in mitochondria and an increased stimulatory effect of FCCP.

The results presented in this study underscore the contribution of mitochondria to cell Ca^{2+} homeostasis in hippocampal and cerebral cortex cells incubated under resting conditions, larger than previously suspected but consistent with the findings in other neuronal and non-neuronal cells (Miller, 1992; Duchen, 1992). Studies in endothelial M4 cells expressing recombinant aequorin targeted to mitochondria have revealed that changes in matrix mitochondrial free Ca^{2+} concentration in response to physiological stimuli that increase $[Ca^{2+}]_i$ are also larger than previously believed (Rizzuto *et al.*, 1992). *In situ* imaging of compartmentalized fura 2 in pancreatoma cells has led to similar conclusions (Glennon *et al.*, 1992). In hippocampal neurons, rhodamine 123 fluorescence in rhodamine 123-rich areas diminishes upon KCl addition (results not shown), suggesting a rapid response of mitochondria to increased $[Ca^{2+}]_i$ involving Ca^{2+} uptake and a $\Delta\psi_{mit}$ decrease.

Our results imply that the global affinity of the mitochondrial Ca^{2+} transport systems *in situ* is adequate to allow Ca^{2+} accumulation from the cytosol at normal resting $[Ca^{2+}]_i$ provided that respiratory substrate supply is not limiting. Indeed, we have found that Ca^{2+} accumulation by mitochondria *in situ* in digitonin permeabilized synaptosomes supplied with glucose, pyruvate, and malate may be observed at calcium concentrations as low as 200 nM, depending on the presence of the known modulators of the uptake and efflux pathways, sodium, ADP, P_i, and polyamines; and as low as 100 nM in intact synaptosomes under resting conditions (Martínez-Serrano and Satrústegui, 1992). Since the mitochondrial Ca^{2+} content in CNS neurons depends upon the supply of exogenous substrates to mitochondria, the actual role of mitochondria in cytosolic calcium buffering awaits further study with appropriately fueled neurons.

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